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Monoclonal Antibodies That Recognize Various Folding States of Pure Human Butyrylcholinesterase Can Immunopurify Butyrylcholinesterase from Human Plasma Stored at Elevated Temperatures

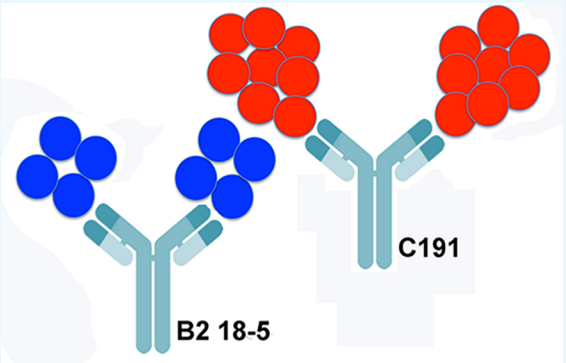
Hong Peng,[†] Thomas A. Blake,[‡] Rudolph C. Johnson,[‡] Alicia J. Dafferner,[†] Stephen Brimijoin,[§] and Oksana Lockridge*[,†]

[†]Eppley Institute, University of Nebraska Medical Center, Omaha, Nebraska 68198-5950, United States

[‡]Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, 4770 Buford Highway, MS F44, Chamblee, Georgia 30341, United States

[§]Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Rochester, Minnesota 55905, United States

ABSTRACT: Human plasma to be analyzed for exposure to cholinesterase inhibitors is stored at 4 °C or lower to prevent denaturation of human butyrylcholinesterase (HuBChE), the biomarker of exposure. Currently published protocols immunopurify HuBChE using antibodies that bind native HuBChE before analysis by mass spectrometry. It is anticipated that the plasma collected from human casualties may be stored nonideally at elevated temperatures of up to 45 °C for days or maybe weeks. At 45 °C, the plasma loses 50% of its HuBChE activity in 8 days and 95% in 40 days. Our goal was to identify a set of monoclonal antibodies that could be used to immunopurify HuBChE from plasma stored at 45 °C. The folding states of pure human HuBChE stored at 4 and 45 °C and boiled at 100 °C were visualized on nondenaturing gels stained with Coomassie blue. Fully active pure HuBChE tetramers had a single band, but pure HuBChE stored at 45 °C had four bands, representing native, partly unfolded, aggregated, and completely denatured, boiled tetramers. The previously described monoclonal B2 18-5 captured native, partly unfolded, and aggregated HuBChE tetramers, whereas a new monoclonal, C191 developed in our laboratory, was found to selectively capture completely denatured, boiled HuBChE. The highest quantity of HuBChE protein was extracted from 45 °C heat-denatured human plasma when HuBChE was immunopurified with a combination of monoclonals B2 18-5 and C191. Using a mixture of these two antibodies in future emergency response assays may increase the capability to confirm exposure to cholinesterase inhibitors.



INTRODUCTION

The current Centers for Disease Control and Prevention protocol for analyzing exposure to cholinesterase inhibitors is based on the fact that organophosphorus toxicants bind irreversibly to human butyrylcholinesterase (HuBChE) in human plasma. Exposure is detected with mass spectrometry by measuring an adduct on the active site serine of HuBChE in the peptide FGESAGAAS.[1−4] HuBChE is a minor component in human plasma having a concentration of 4 mg/L against a background protein concentration of 60 000 mg/L. The first step in the published protocols selectively extracts HuBChE from plasma by binding HuBChE to an immobilized antiHuBChE monoclonal antibody.

It is anticipated that some plasma samples will have been stored under conditions that denature HuBChE (i.e., at elevated temperatures for prolonged periods). Monoclonals that recognize denatured HuBChE would enhance the sensitivity of the immunopurification-based assay for confirming exposure to cholinesterase inhibitors. Our goal was to develop a set of monoclonals that could be used for immunopurifying heat-inactivated, denatured HuBChE.

We started our study by asking what happens to pure tetrameric HuBChE when it is stored at 4 and 45 °C and boiled at 100 °C. We expected to find irreversible loss of activity in HuBChE exposed to elevated temperatures, but we did not know whether elevated temperatures caused the protein to precipitate or the HuBChE tetramer to dissociate into dimers and monomers or to fragment by breaking peptide bonds. We also did not know which immobilized monoclonals would serve to immunopurify heat-denatured HuBChE. Having learned what to expect from our studies of pure HuBChE, we applied

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the information to human plasma samples stored at elevated temperatures for prolonged times.

In this study, we use the term "boiled HuBChE" for heat-denatured HuBChE. There are many ways to denature HuBChE, including treatment with high or low temperatures, organic solvents, high pressure,[5] drying, aqueous solvents with an extremely low or high pH, cross-linking agents such as glutaraldehyde, chaotropic agents such as urea and guanidine hydrochloride, digestion with proteases, disulfide bond-reducing agents, and amino acid-modifying agents such as fluorescent IRDye 800CW.[6] Each of these can cause complete loss of enzyme activity, so that the treated enzyme can be described as "completely denatured". However, the structural change in the protein may be unique to the treatment. Therefore, we define heat-denatured HuBChE as "boiled" rather than as "completely denatured".

MATERIALS AND METHODS

Materials. The following were from Millipore, Billerica, MA: 0.22 μm presterilized disposable filtration system (Stericup SCGVU11RE); Amicon Ultra-15 centrifugal filter 10 000 NMWL (UFC 901024); Durapore PVDF 0.45 μm centrifugal filter (UFC30HV00). The following were from Bio-Rad Laboratories Inc., Hercules, CA: Immun-Blot PVDF membrane (162-0177); Clarity Western ECL substrate (170-5060); Precast 4−20% gradient gels (456-1094); Mini-Protean Tetra Cell (165-8003). Protein G agarose was from Protein Mods LLC, Madison, WI, (product code PGGH). CNBr-activated Sepharose 4B powder was from Amersham Biosciences AB (GE Healthcare, Pittsburgh, PA, 17-0981-01). Q-Ceramic HyperD F sorbent was from Pall Corp., Port Washington, NY (cat# 20066-56). Hupresin is a new affinity gel manufactured by Emilie David at Chemforase, Mont-Saint-Aignan, France. 4−30% gradient gels, with a 4% stacking gel, were poured in a vertical slab electrophoresis unit from Hoefer Scientific Instruments, San Francisco, CA (SE 600). Frozen Cohn fraction IV-4 was from Grifols Therapeutics Inc., Clayton, NC.

Antibodies. Monoclonal B2 18-5 was previously produced against native HuBChE in mice.[7] The heavy and light chain nucleotides of B2 18-5 were sequenced, cloned into expression vectors, expressed in a stable Chinese hamster ovary cell line, and the antibody purified by Syd Labs, Natick, MA.[8] B2 18-5 efficiently immunopurifies HuBChE from plasma stored at 4 or −20 °C.[8] Antimouse IgG conjugated to horse radish peroxidase was from Cell Signaling, cat# 7076.

Monoclonal C191 was created at the University of Nebraska Medical Center (UNMC) by injecting mice with denatured, recombinant human HuBChE expressed in Chinese hamster ovary cells. The recombinant HuBChE had been denatured and reduced by boiling in 1% sodium dodecyl sulfate (SDS) containing 5% mercaptoethanol before it was injected into mice with an adjuvant. The coding sequence had been modified to delete the 44 amino acids in the tetramerization domain at the C-terminus and to reduce the number of *N*-glycans from nine to five. The amino acid sequence and crystal structure of the truncated HuBChE protein can be found in Protein Data Bank entry 1P0I.[9]

Purification of Monoclonal C191 on Protein G Agarose. Monoclonal C191 was purified from mouse hybridoma cell culture medium on Protein G agarose using 0.1 M sodium phosphate pH 7.5 for equilibration and washing, 0.1 M citric acid pH 2.6 for elution, and 1 M sodium phosphate pH 9.5 for neutralization. The buffer was changed to phosphate-buffered saline (PBS), and the protein concentrated to 5 mg/mL by diafiltration through an Amicon centrifugal filter. Tris and azide were avoided because they interfere with conjugation of antibody to CNBr-activated Sepharose.

Cross-linking Monoclonals C191 and B2 18-5 to CNBr-Activated Sepharose. A 5 mg aliquot of each monoclonal in buffer free of Tris and azide was cross-linked to 3.5 mL Sepharose that had been swollen from 1 g of CNBr-activated Sepharose 4B powder. The dry Sepharose was hydrated and washed with 13 mL of ice-cold 1 mM HCl followed by 13 mL of ice-cold coupling buffer, 0.15 M $NaHCO_3$, 0.5 M NaCl pH 8. The beads were pelleted by centrifugation, and the liquid discarded before addition of 5 mg monoclonal in 1 mL PBS and 1 mL coupling buffer. The tubes were rotated at room temperature for 20 h. It was estimated that 99% of the antibody bound to Sepharose as calculated from the observed absorbance of 0.03 at 280 nm in the supernatant liquid. The beads were washed twice with the coupling buffer, once with Tris-buffered saline containing 0.2% Tween-20 and twice with PBS 0.05% sodium azide. The 3.5 mL beads coupled to 5 mg monoclonal were stored in a total volume of 15 mL PBS 0.05% azide at 4 °C. A 0.1 mL suspension has 33 μg monoclonal bound to 23 μL beads.

Pure HuBChE. Cohn fraction IV-4, a byproduct of the human plasma fractionation industry, was the starting material for purifying large quantities of HuBChE.[10,11] HuBChE was purified from frozen Cohn fraction IV-4 paste using anion-exchange chromatography on Q-Ceramic HyperD F sorbent followed by affinity chromatography on hupresin. Hupresin was recently introduced as a new affinity gel for purification of rHuBChE expressed in insect cells.[12]

Human Plasma. Pooled human plasma from the Nebraska Medical Center Blood Bank contained acid citrate dextrose anticoagulant.

Heat Inactivation of Pure Human HuBChE. Pure plasma-derived HuBChE was diluted with PBS 0.1% azide to an activity of 27.5 u/mL and a protein concentration of 0.05 mg/mL. 1 mL aliquots of HuBChE were stored at 4, 24, 37, and 45 °C. The 100 °C sample was prepared by heating a 1 mL aliquot in a boiling water bath for 5 min, a procedure that completely destroyed HuBChE activity with butyrylthiocholine. The boiled HuBChE was stored at 4 °C. The stability of HuBChE activity was measured as a function of time of storage in Figure 1.

Inactivation of HuBChE in Human Plasma at 45 °C. Human plasma was incubated at 45 °C (113 °F). The loss of HuBChE activity in plasma was measured as a function of time at 45 °C.

HuBChE Activity. Activity was measured with 1 mM butyrylthiocholine iodide in 0.1 M potassium phosphate pH 7.0 containing 0.5 mM 5,5′-dithiobis(2-nitrobenzoic acid) at 25 °C by recording the increase in absorbance at 412 nm on a Gilford spectrophotometer interfaced to MacLab (ADInstruments, Colorado Springs, CO). The increase in absorbance between 10 and 40 s was used to calculate units per milliliter where a unit (u) of activity is micromoles hydrolyzed per minute. The extinction coefficient for the calculation was 13.6 mM^{-1} cm^{-1}.[13]

Gel Shift Assay. The complex formed between monoclonal B2 18-5 and heat-inactivated pure HuBChE was visualized on a nondenaturing 4−30% gradient polyacrylamide gel, overlaid with a 4% stacking gel, and stained with Coomassie blue. The samples were prepared for nondenaturing gel electrophoresis

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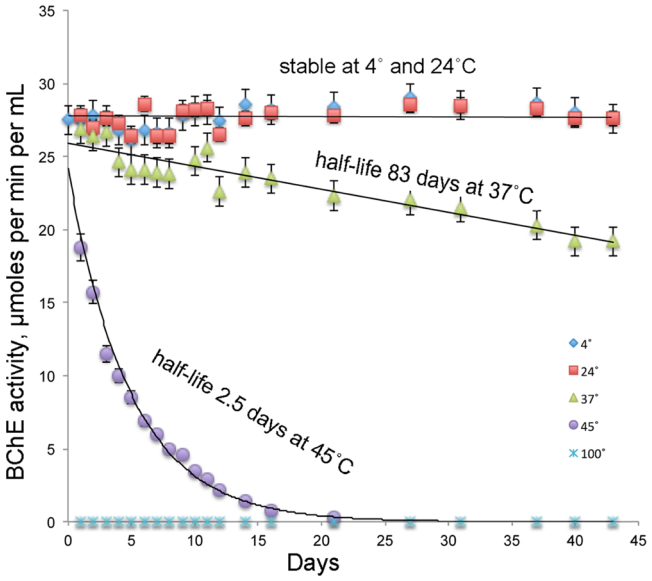

Figure 1. Stability of pure HuBChE in PBS 10 mM azide. Pure HuBChE with an activity at time zero of 27.5 units/mL and a protein concentration of 0.055 mg/mL was incubated in 1 mL aliquots at various temperatures. HuBChE activity was monitored for 43 days. Triplicate assays were performed at each time point.

by incubating 10 μL of pure HuBChE (0.48 mg/mL) with either 5 or 2 μL of monoclonal B2 18-5 (0.91 mg/mL) adjusted to 15 μL with PBS, at room temperature for 2 h. A 15 μL aliquot of 60% glycerol, 0.1% bromphenol blue was added to each tube before the samples were loaded on a nondenaturing gel. Electrophoresis at 300 V (constant voltage) was conducted for 24 h at 4 °C in a Hoefer gel apparatus. The gel shift assay is shown in Figure 3.

Activity Stained Gel. A nondenaturing 4−30% gradient gel was stained for HuBChE activity by the method of Karnovsky and Roots,[14] as shown in Figure 4.

Capture of Heat-Inactivated Pure HuBChE by Immobilized Monoclonals. The selective removal of partly unfolded HuBChE by immobilized B2 18-5 and of completely denatured HuBChE by immobilized C191 was visualized on a nondenaturing 4−30% gradient gel stained with Coomassie blue. The Sepharose beads in 0.3 mL suspension of immobilized B2 18-5 or C191 occupied a volume of 70 μL after the liquid phase was discarded. Pure HuBChE in PBS 0.1% azide at a concentration of 0.48 mg/mL had been stored at 4 or 45 °C or boiled in a 100 °C water bath and then stored at 4 °C. A 30 μL aliquot of pure HuBChE was added to the beads and mixed in a rotating mixer for 5 h at room temperature. A 10 μL aliquot of the clear supernatant was removed from each incubation mixture for gel electrophoresis on a nondenaturing gradient gel stained with Coomassie blue to visualize bands not captured by the monoclonals. The method was used in Figure 6.

The beads were washed with detergent-containing buffer (PBS with 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 20 mM ethylenediaminetetraacetic acid (EDTA), 10 mM sodium azide, HCl adjusted to pH 6.5), and the bound protein was released with 50% acetonitrile 1% trifluoroacetyl (TFA). The method was used for the SDS gel in Figure 7.

Dynamic Light Scattering. The size of the pure HuBChE samples stored at 4, 45 °C or boiled at 100 °C was measured on the Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Malvern, UK) in disposable folded capillary cells (DTS1070) for 0.1 mL aliquots of 0.5 mg/mL of HuBChE in PBS. Measurements at 25 °C were repeated three times for a total of 42 scans per sample.

Capture of Heat-Inactivated HuBChE from Human Plasma Stored at 45 °C. Human plasma (1 mL) stored at 45 °C for 14 days lost 60% of its original HuBChE activity and developed a heavy precipitate. The liquid and solid phases were treated separately. The 0.85 mL plasma in the liquid phase was clarified through a 0.45 μm nylon syringe filter before a 0.7 mL aliquot of the filtered plasma was incubated with a mixture of 0.2 mL immobilized B2 18-5 and 0.2 mL immobilized C191. It was estimated that each 0.2 mL suspension contained 66 μg monoclonals conjugated to 46 μL beads. The samples were rotated at room temperature overnight. The beads were transferred to a 0.45 μm centrifugal filter where they were washed three times with PBS containing 0.05% Tween-20 and two times with water.

HuBChE in the 45 °C plasma pellet was partly solubilized with 400 μL of 1% Triton X-100 in PBS at 37 °C overnight. The supernatant was diluted 10-fold with PBS before addition of 0.2 mL immobilized B2 18-5 and 0.2 mL immobilized C191 suspensions. The samples were mixed overnight at room temperature and washed as above in preparation for Western blotting.

Western Blot. HuBChE immunopurified from human plasma was visualized on Western blots in Figure 8. Proteins were extracted from immobilized B2 18-5 and C191 monoclonals with 200 μL of 50% acetonitrile 0.1% trifluoroacetic acid. The extract was dried, suspended in 5 μL water and 5 μL Laemmli buffer (0.125 M TrisCl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol at pH 6.8, 0.01% bromophenol blue), heated in a boiling water bath for 3 min, and loaded on an 8% SDS polyacrylamide gel. Following electrophoresis, proteins were transferred to a PVDF membrane in 25 mM Tris, 192 mM glycine pH 8.3 at 4 °C for 1 h at 350 mA. The membrane was blocked in 5% nonfat dry milk in 50 mM Tris pH 7.4, 150 mM NaCl, 0.02% Tween-20 (TBST with 5% milk) for 1 h, followed by washing three times with TBST. The blot was incubated at 4 °C overnight with 20 μL monoclonal antibody C191 culture medium diluted in 10 mL PBS containing 5% milk. The membrane was washed three times with TBST and incubated 1 h at room temperature with 1 μL antimouse IgG linked to horse radish peroxidase in 5 mL of TBST containing 5% milk. The membrane was washed with TBST. Bands were visualized by treating the blot with Clarity Western ECL substrate and detecting the emitted light on X-ray film. Known quantities of pure HuBChE were loaded on the SDS gel to serve as controls.

LC-MS/MS on the 6600 Triple-TOF Mass Spectrometer (AB Sciex). Mass spectrometry was used to demonstrate that monoclonal C191 recognizes denatured HuBChE immunopurified from 45 °C heat-treated plasma. Following incubation with plasma, the beads were transferred to a 0.45 μm centrifugal filter where they were washed with a detergent-containing buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 20 mM EDTA, and 10 mM NaN_3 in PBS adjusted to pH 6.5) and desalted by washing with water. Proteins captured by immobilized monoclonal C191 were released with 100 μL of 50% acetonitrile 0.1% TFA, dried, dissolved in 20 mM ammonium bicarbonate, and digested with trypsin. A 5 μL aliquot of the digest was analyzed using liquid chromatography

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(LC)−mass spectrometry (MS)/MS. Details of the LC-MS/MS system have been previously described.[15]

Amino Acid Sequence of Monoclonals C191 and B2 18-5. The nucleotide and deduced amino acid sequences of the light and heavy chains of monoclonals C191 and B2 18-5 were determined by Syd Labs (Natick, MA) from the mRNA in hybridoma cells. The reported sequences were validated by testing the specificity of the recombinant monoclonals produced by transient expression in mammalian cells.

RESULTS

Stability of Pure HuBChE in PBS at Various Temperatures. The goal was to identify a temperature and storage time that converts HuBChE to the type of unfolded, inactive molecule present in plasma that has been stored at elevated temperatures. Figure 1 shows that solutions of pure HuBChE at a concentration of 0.05 mg/mL were stable at 4 and 24 °C for at least 43 days. Storage at 37 °C resulted in a slow loss of HuBChE activity with time; the activity after 14 days at 37 °C was 87% and after 43 days was 70% of the initial activity. Storage at 45 °C resulted in a loss of 50% of the HuBChE activity in 2.5 days and 95% loss in 14 days. Pure HuBChE that had been denatured in a boiling water bath (100 °C) had no activity following heat treatment.

Plasma Stored at Elevated Temperatures. Human plasma in anticoagulant citrate dextrose lost HuBChE activity when the plasma was incubated at the elevated temperature of 45 °C (113 °F). Figure 2 shows that after 8 days at 45 °C, the

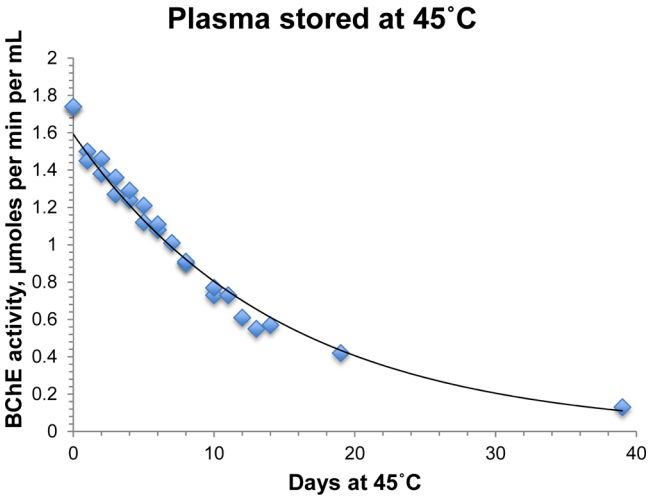

Figure 2. Loss of HuBChE activity in human plasma stored at 45 °C. Values for triplicate assays are too close to show up in error bars.

HuBChE activity in plasma was 50% of its initial activity. After 19 days at 45 °C, the plasma lost 75% of its initial activity, and after 39 days, it lost 93% of its initial HuBChE activity.

After a chemical emergency, researchers may be required to analyze plasma samples that have been stored at temperatures as high as 45 °C. The methods published to date use HuBChE in plasma samples that are stored frozen or refrigerated. The loss of HuBChE activity in samples stored at 45 °C suggests the HuBChE protein has undergone unfolding and that the unfolded HuBChE protein may require the use of an antibody directed to denatured HuBChE.

Folding States of HuBChE. Antibody binding and gel electrophoresis experiments described below showed that pure HuBChE incubated at 45 °C contains inactive HuBChE structures in addition to fully active, native HuBChE. A partly unfolded HuBChE structure and an aggregated HuBChE structure are recognized by monoclonal B2 18-5, the same monoclonal that captures native HuBChE. The completely denatured HuBChE is recognized by monoclonal C191. Table 1 presents the monoclonals that distinguish the folding states of

Table 1. HuBChE Folding States Distinguished by Antibody Binding

monoclonal	HuBChE folding state	method for producing folding state
B2 18-5	native HuBChE	store plasma or pure HuBChE at 4 °C
B2 18-5	partly unfolded HuBChE; aggregated HuBChE	store pure HuBChE in PBS at 45 °C for 3−14 days; store human plasma at 45 °C for 5−40 days[a]
C191	completely denatured HuBChE with aggregates	heat pure HuBChE at 100 °C

[a]Storage at 45 °C produces a mixture of HuBChE folding states including native, partly unfolded, aggregated, and completely unfolded HuBChE structures.

heat-inactivated HuBChE and the methods to produce these folding states. Table 1 does not include plasma heated to 100 °C because plasma congeals to a solid form at 100 °C, a condition not amenable to antibody-binding assays.

Gel Shift Assay. The gel shift assay in Figure 3 demonstrates that storage of pure HuBChE in PBS azide at 45 °C for 9 days with 55% loss of activity creates HuBChE structures that differ from native and boiled HuBChE. The nondenaturing gel compares the migration of pure HuBChE stored at 4 °C (lane 1), 45 °C (lane 2), and 100 °C (lane 3). The HuBChE tetramer stored at 4 °C has a single band (lane 1), whereas the 45 °C heat-inactivated HuBChE (lane 2) has bands for native and partly unfolded, a faint band corresponding to boiled HuBChE, and a diffuse band near the top of the gel for aggregated HuBChE. The boiled HuBChE (lane 3) has an intense band that migrates further toward the positive pole than the native HuBChE tetramer, and in addition has diffuse aggregated bands near the top of the gel and a faint band slightly above the native tetramer band. The fastest migrating band in lane 2 (which is barely detectable in lane 2 but visible in lane 6) is at the position of boiled HuBChE in lane 3, suggesting that some of the 45 °C heat-inactivated HuBChE is completely denatured.

Figure 3 lane 5 shows that 4.5 μg of monoclonal B2 18-5 shifts the migration of 4.4 μg native HuBChE to slower moving bands near the top of the gel. This ratio of monoclonal to HuBChE protein captures all the HuBChE. The IgG monoclonal has two antigen-binding domains per molecule. The HuBChE tetramer has four antibody binding sites per tetramer, enabling it to form a polymer-like complex that cross-links several HuBChE tetramers through interaction with two or more monoclonals. The bands at the top of lanes 5, 6, 8, and 9 probably represent such high molecular weight complexes. When the HuBChE (4.4 μg) concentration exceeds the antibody concentration (1.8 μg) as in lane 8, the cross-linking may be minimized to yield lower molecular weight complexes where one HuBChE tetramer binds only one or two antibodies. The two bands in lane 8 that are missing from lane 5 may represent one HuBChE tetramer bound to one or two antibodies.

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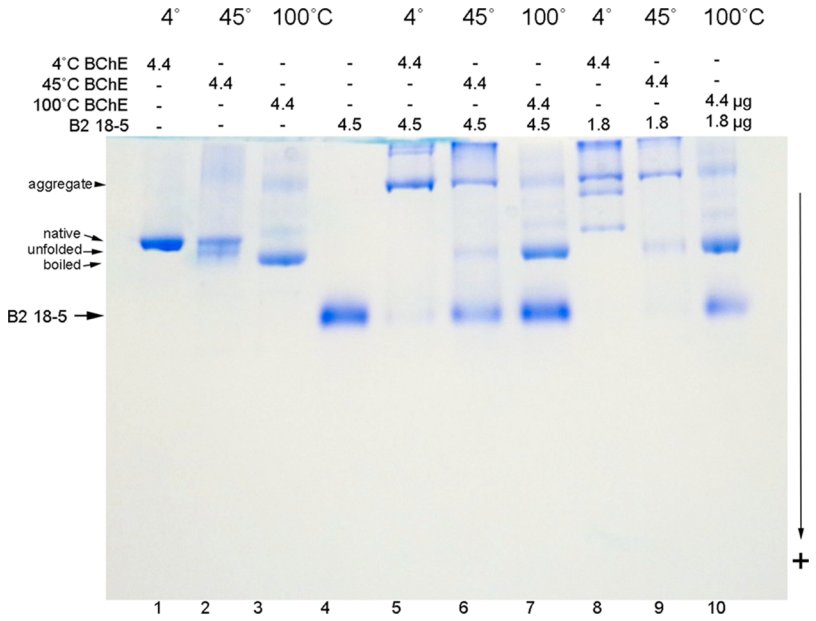

Figure 3. Gel shift assay on a nondenaturing 4−30% gradient gel with 4% stacking gel, stained with Coomassie blue. (1) Pure HuBChE tetramer stored at 4 °C has a single band. (2) Pure HuBChE stored at 45 °C has a band for native HuBChE, a faster migrating band for unfolded HuBChE, a barely detectable band at the level of boiled HuBChE, and a diffuse slow-moving band for aggregated forms. (3) Boiled pure HuBChE has a prominent band that migrates faster than the unfolded band in lane 2; in addition boiled HuBChE has several diffuse aggregated bands. (4) Monoclonal B2 18-5 migrates faster toward the positive pole than HuBChE. (5) Monoclonal B2 18-5 forms a complex with native 4 °C HuBChE seen as prominent bands near the top of the gel; lane 5 shows no unbound HuBChE. (6) B2 18-5 forms a complex with native and partially unfolded HuBChE in the 45 °C sample but does not form a complex with boiled HuBChE. (7) B2 18-5 does not bind any of the protein forms in boiled HuBChE. (8) 1.8 μg monoclonal B2 18-5 is saturated by 4.4 μg native HuBChE, forming additional complexes compared to those in lane 5. (9) 1.8 μg antibody captured all of the native, partially unfolded, and aggregated HuBChE in the 45 °C sample but not the structure representing boiled HuBChE. (10) 1.8 μg antibody did not capture boiled HuBChE. The direction of migration was toward the positive pole, indicated by the + sign.

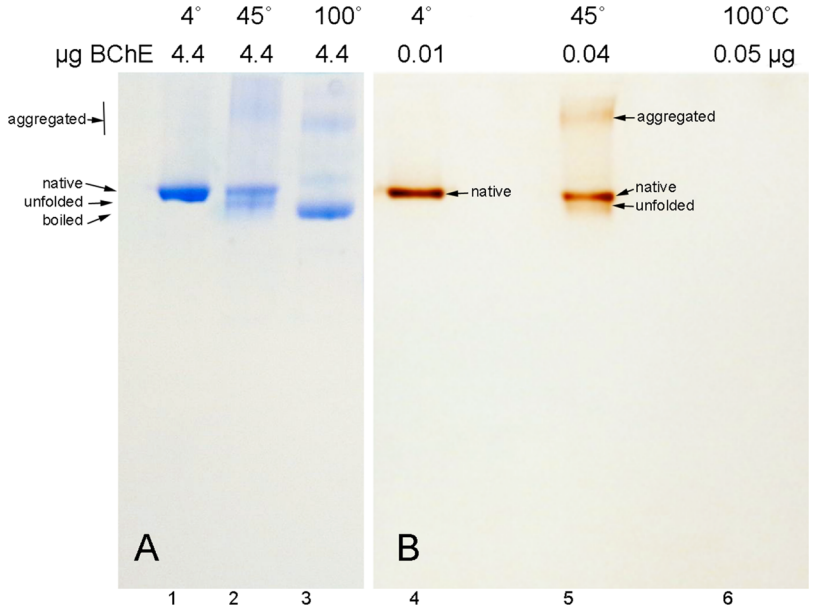

Figure 4. Low HuBChE activity in unfolded HuBChE. The nondenaturing gradient gel in panel A stained with Coomassie blue was loaded with 4.4 μg HuBChE protein per lane. The gel in panel B stained for HuBChE activity was loaded with 0.005 units of HuBChE activity, which required 0.01 μg of HuBChE protein for the 4 °C sample and 0.04 μg HuBChE protein for the 45 °C sample. The 100 °C sample had no activity.

Figure 3 lane 6 shows that 4.5 μg of monoclonal B2 18-5 shifts the migration of bands corresponding to native and partly unfolded tetramers in 45 °C heat-inactivated HuBChE, but does not shift the band that corresponds to boiled HuBChE. Lane 7 confirms that B2 18-5 does not recognize completely denatured HuBChE. We conclude that native, partly unfolded, and aggregated HuBChE structures bind to B2 18-5. However, completely denatured HuBChE does not bind to B2 18-5.

The gel in Figure 3 has a stacking gel on top of a 4−30% acrylamide gradient. Electrophoresis was performed for 24 h at 300 V constant voltage in a Hoefer apparatus. This system separated the unbound antibody (lane 4) from unbound

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HuBChE tetramers (lane 1). However, when the same samples were run on a precast 4−20% gradient gel (no stacking gel), the unbound HuBChE migrated to the same position as the unbound antibody.

Low Activity in the Partly Unfolded HuBChE. The epitope for binding monoclonal B2 18-5 was present in native, partly unfolded HuBChE and in aggregated HuBChE produced at 45 °C but was absent in completely denatured HuBChE. This means that the epitope is formed by a three-dimensional arrangement of residues, rather than by adjacent residues in a linear segment. To determine which folding states of HuBChE have HuBChE activity, the samples were subjected to nondenaturing gel electrophoresis in Figure 4B, where the gel was stained for HuBChE activity. For comparison, the gel in Figure 4A was stained with Coomassie blue to show the location of HuBChE bands in pure HuBChE stored at 4, 45 °C and boiled at 100 °C. Lanes 4 and 5 in Figure 4B show an intense band of activity for the native HuBChE tetramer. The 45 °C sample in lane 5 has faint bands of activity for the unfolded and aggregated HuBChE. The boiled HuBChE sample in lane 6 had no activity. It was concluded that the partly unfolded HuBChE and aggregated HuBChE structures produced by storage at 45 °C have a very low level of HuBChE activity. Furthermore, the aggregated forms that have HuBChE activity in the sample stored at 45 °C have a different structure than the aggregated forms in boiled HuBChE that have no HuBChE activity.

HuBChE Aggregates to 30 times Normal Size. The size of pure HuBChE stored at elevated temperatures was estimated by dynamic light scattering (DLS). The mean diameter of the 340 kDa HuBChE tetramer was 20 nm for HuBChE stored at 4 °C (see Figure 5), 18 nm for HuBChE stored at 45 °C, and 17 nm for boiled HuBChE. The broad peak for pure HuBChE is attributed to microheterogeneity of the 36 asparagine-linked glycans in the 340 kDa tetramer. The storage of pure HuBChE in PBS azide for 9 days at 45 °C converted 20% of the HuBChE to aggregates with a diameter of 490 nm. Boiling converted 5% of HuBChE to aggregates with a diameter of 100 nm and 45% of HuBChE to aggregates with a mean diameter of 620 nm.

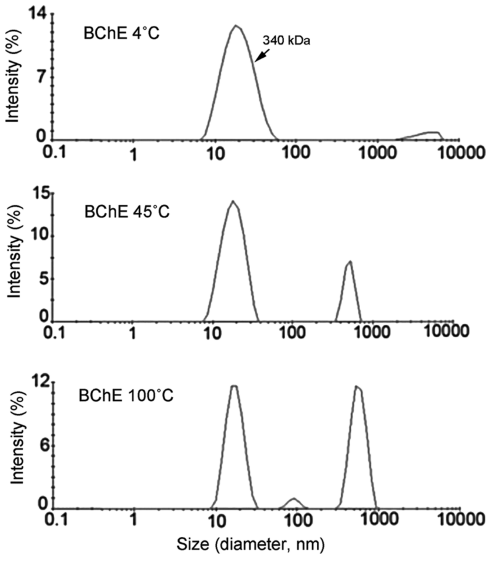

Figure 5. DLS of pure HuBChE stored at 4 and 45 °C and boiled at 100 °C. The 4 °C sample consists predominantly of 340 kDa HuBChE tetramers with an average diameter of 20 nm. Heat treatment produces aggregates with an average diameter of 500−600 nm.

DLS did not resolve HuBChE into the bands visualized by nondenaturing gel electrophoresis. For example, the most intense band in boiled HuBChE (Figure 3, lane 3) migrates further than the band in 4 °C HuBChE (Figure 3, lane 1). This difference is not apparent in Figure 5. Coomassie blue staining of the nondenaturing gel in Figure 3 showed only faint bands for aggregated HuBChE in the 100 °C sample, whereas DLS indicated that 50% of the HuBChE had aggregated. Both methods agree that heating under our conditions produces high molecular weight aggregates, but does not fragment the HuBChE protein into smaller peptides.

Nondenaturing Gel Shows HuBChE Bands Not Captured by B2 18-5 and C191. A second approach for demonstrating the specificity of monoclonals B2 18-5 and C191 used nondenaturing gel electrophoresis to show the bands of pure HuBChE that failed to bind to immobilized antibodies. In Figure 6, lane 4 is blank, which means all the native 4 °C pure HuBChE was captured by B2 18-5. In lane 5 for 45 °C heat-inactivated HuBChE, all bands disappeared (compare lanes 2 and 5), except for a faint band at the position of boiled HuBChE. This means only native HuBChE, partly unfolded, and aggregated HuBChE are recognized by B2 18-5. The bands for boiled HuBChE are present in lane 6, indicating that B2 18-5 does not recognize completely denatured HuBChE. By contrast, lane 9 is nearly blank, which shows that C191 captured boiled HuBChE. However, C191 did not bind native HuBChE (lane 7) and did not bind the bands in 45 °C HuBChE for native and partly unfolded HuBChE (lane 8). The nondenaturing gel in Figure 6 shows that 33 μg immobilized B2 18-5 captured 100% of the 5 μg of native, partially unfolded, and aggregated HuBChE in 4 and 45 °C samples, whereas 33 μg immobilized C191 captured 99% of the 5 μg boiled HuBChE. In a previous study,[8] we have shown that 20 μg immobilized monoclonal B2 18-5 captures 97% of the HuBChE in 0.5 mL human plasma. We expect that B2 18-5 and C191 monoclonals used together will immunopurify the maximum amount of HuBChE from human plasma stored at elevated temperatures.

SDS Gel Confirms the Capture of Native, Partly Unfolded, and Aggregated HuBChE by B2 18-5 and the Capture of Completely Unfolded HuBChE by C191. Pure HuBChE at a concentration of 0.48 mg/mL in PBS azide lost no activity at 4 °C, lost 60% activity in 15 days at 45 °C, and lost 100% activity in a boiling water bath. The heat-treated pure HuBChE remained in solution and did not precipitate. Immobilized monoclonals B2 18-5 and C191 (100 μg monoclonal bound to 70 μL Sepharose beads) were incubated with 13 μg of pure HuBChE in 30 μL PBS for 5 h at room temperature. The beads were washed with detergent-containing buffer, and the bound protein was released with 50% acetonitrile 1% TFA. Bound HuBChE released from the immobilized monoclonals is visualized in Figure 7. The SDS gel shows that HuBChE heated to 45 or 100 °C has the same monomer molecular weight of 85 kDa and dimer of 170 kDa as HuBChE stored at 4 °C. Thus, heat treatment did not break peptide bonds. HuBChE (lane 1) stored at 4 °C has a band at 85 kDa, indicating that native HuBChE was bound by monoclonal B2 18-5. However, there is no HuBChE band in lane 4, indicating that monoclonal C191 did not bind native HuBChE. The bands for HuBChE are present in lanes 2 and 5, indicating that HuBChE stored at 45 °C contains HuBChE

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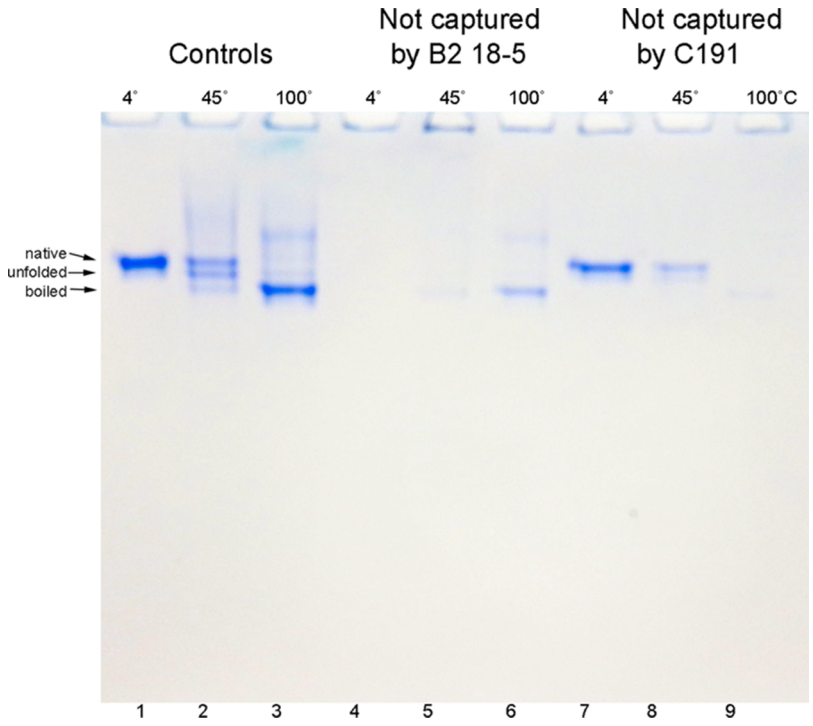

Figure 6. Nondenaturing gel electrophoresis stained with Coomassie blue. Pure HuBChE stored at 4 and 45 °C or boiled was incubated with immobilized B2 18-5 or with immobilized C191. Unbound protein was loaded on the gel. Lanes 1−3 were loaded with 5 μg pure HuBChE. Lanes 4−9 show the unbound HuBChE remaining in solution after incubation of 5 μg HuBChE with 33 μg immobilized monoclonal.

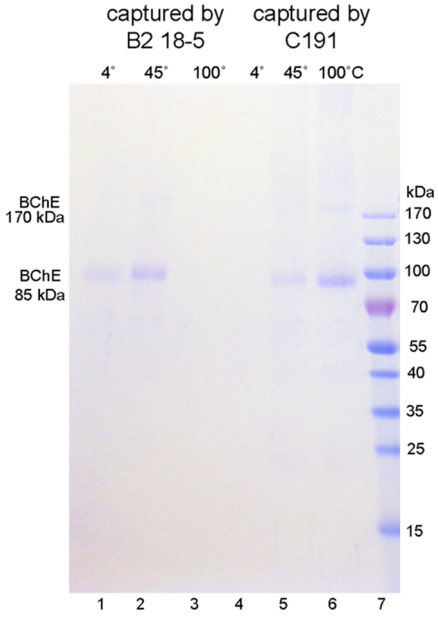

Figure 7. HuBChE captured by immobilized monoclonals was released from binding and visualized on an SDS gel stained with Coomassie blue. HuBChE stored at 4 °C was captured by B2 18-5 (lane 1) but not by C191 (lane 4). HuBChE stored at 45 °C was captured by B2 18-5 (lane 2) and C191 (lane 5). Boiled HuBChE was not captured by B2 18-5 (lane 3) but was captured by C191 (lane 6).

folding states captured by both B2 18-5 and C191 monoclonals. There is no HuBChE band in lane 3, indicating that the boiled HuBChE is not recognized by B2 18-5. However, there is a HuBChE band in lane 6, indicating that monoclonal C191 binds denatured HuBChE. These results confirm the binding specificity of B2 18-5 for native, partly unfolded, and aggregated HuBChE, whereas C191 specifically binds completely denatured HuBChE. All folding states—native, partly unfolded, aggregates, and completely unfolded—are present in HuBChE stored at 45 °C. The use of both antibodies together is expected to yield the highest amount of immunopurified HuBChE.

Monoclonals B2 18-5 and C191 Immunopurify HuBChE from 45 °C Heat-Inactivated Plasma. Having established that heat-denatured pure HuBChE remains in solution, and that B2 18-5 and C191 immunopurify heat-denatured pure HuBChE, we tested these monoclonals for their ability to immunopurify HuBChE from human plasma stored at 45 °C. Western blotting was used to detect HuBChE immunopurified from plasma. Immobilized monoclonals B2 18-5 and C191 were incubated with control plasma stored at 4 °C or with the supernatant in 45 °C heat-inactivated plasma. The plasma stored at 45 °C developed a heavy precipitate. The 4 °C control plasma contained no pellet. The possibility was tested that HuBChE was trapped in the plasma precipitate. The pellet was extracted with Triton X-100, and the diluted extract was incubated with immobilized B2 18-5 and C191. The Western blot in Figure 8A for control plasma (lane 4) indicates a recovery of about 0.5 μg HuBChE from 1 mL control plasma stored at 4 °C. HuBChE activity in the control plasma was 1.8 u/mL, which corresponds to 2.5 μg of HuBChE protein. A recovery of 20% HuBChE protein is therefore expected from heat-inactivated plasma processed through the immunopurification and Western blotting steps.

The Western blot in Figure 8B shows HuBChE immunopurified from plasma stored at 45 °C for 3 weeks. After 1 mL plasma had been separated from the pellet and filtered, only 0.7 mL of plasma remained for incubation with immobilized antibodies. The band intensities in lane 8 correspond to a recovery of about 0.2 μg of HuBChE protein from 0.7 mL plasma, a percent recovery similar to that for control plasma in

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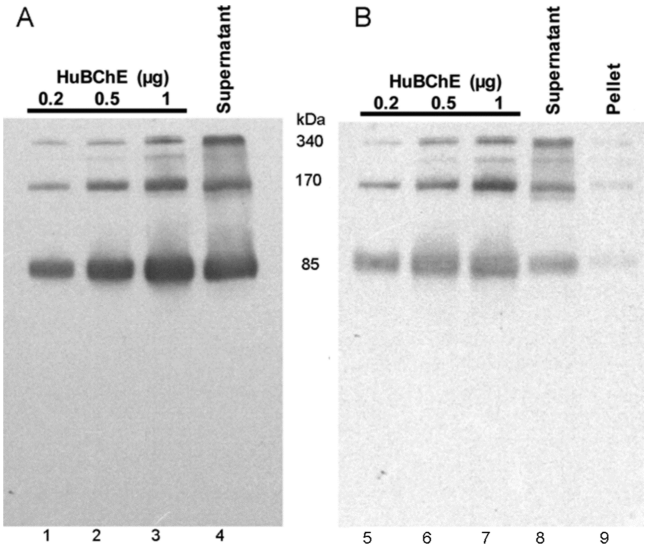

Figure 8. (A) Western blot for HuBChE immunopurified from 1 mL control 4 °C plasma (lane 4) by an equal mixture of immobilized B2 18-5 and C191 indicates a recovery of 20% HuBChE protein, which is based on the comparison to the pure HuBChE standards in lanes 1−3. (B) Western blot for HuBChE immunopurified from 45 °C heat-inactivated plasma by a 1:1 mixture of immobilized B2 18-5 and C191. More HuBChE was recovered from the supernatant (lane 8) than the pellet (lane 9).

lane 4. Comparison of band intensities for HuBChE immunopurified from the plasma supernatant (lane 8) and the pellet (lane 9) shows that the supernatant contained most of the HuBChE protein. This result for plasma is consistent with the observation that pure HuBChE remained in solution after prolonged incubation at 45 °C and after denaturation in a boiling water bath.

Plasma stored at 45 °C for 2 weeks with a 67% loss of HuBChE activity was immunopurified with immobilized B2 18-5. The extracted protein gave a good HuBChE signal on a Western blot, corresponding to about 0.2 μg of protein. The same sample immunopurified with C191 gave a weak signal, corresponding to 0.02 μg of HuBChE. After 3 weeks at 45 °C, when plasma had lost 80% of its HuBChE activity, the two monoclonals captured equal amounts of HuBChE proteins.

Mass Spectrometry Confirms that C191 Immunopurifies HuBChE from Heat-Denatured Plasma. Human plasma stored at 45 °C for 3 weeks with 80% loss of HuBChE activity was incubated with immobilized C191 monoclonal. Protein released from the washed beads was digested with trypsin and analyzed by LC-MS/MS on a 6600 Triple-TOF mass spectrometer. Protein Pilot software identified the HuBChE peptides colored green and red in Figure 9. Of the 574 amino acids in the mature HuBChE protein, 217 amino acids were identified in Figure 9 for a coverage of 38%. Mass spectrometry confirmed the finding from Western blots that monoclonal C191 immunopurifies HuBChE from heat-inactivated plasma. This means that some of the HuBChE in plasma stored at 45 °C is completely denatured.

Amino Acid Sequences of Monoclonals B2 18-5 and C191. The amino acid sequences of monoclonals B2 18-5 and C191 in Figure 10 were deduced from the nucleotide sequences

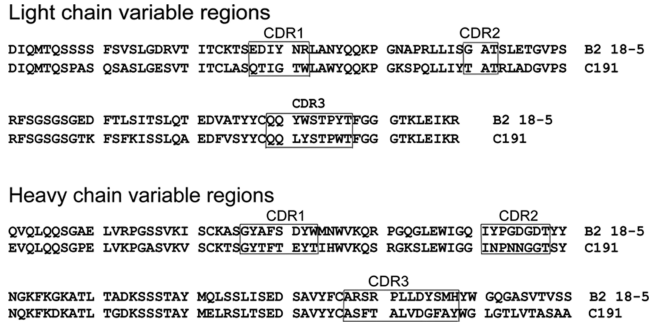

Figure 10. Amino acid sequences of the light and heavy chain variable regions of mouse anti-HuBChE monoclonal antibodies B2 18-5, isotype IgG2b κ, and C191, IgG1 κ. The CDRs were defined using VBASE2 http://www.vbase2.org/.

of the cDNA. The complementarity-determining regions (CDRs) are boxed because they are the hypervariable domains that determine antibody-binding specificity.

The nucleotide and amino acid sequences for monoclonals B2 18-5[8] and C191 have been deposited in the National Center for Biotechnology Information database under the accession numbers listed in Table 2. Knowledge of the sequences of monoclonals is valuable because it allows one to produce the monoclonals using recombinant DNA techniques when the hybridoma cells are unavailable or when the antibody genes in the hybridoma cells have undergone deleterious mutations.

Table 2. Accession Numbers for Monoclonals B2 18-5 and C191

mouse monoclonal		isotype	NCBI accession number
B2 18-5	heavy chain	IgG2b	KT189143
B2 18-5	light chain	κ	KT189144
C191	heavy chain	IgG1	KX278822
C191	light chain	κ	KX278823

DISCUSSION

Antibodies for Immunopurification of Heat-Inactivated HuBChE from Plasma. Monoclonals B2 18-5 and C191 can be used to immunopurify heat-inactivated HuBChE in human plasma that has been stored at 45 °C. Monoclonal B2 18-5 recognizes native HuBChE and two HuBChE con-

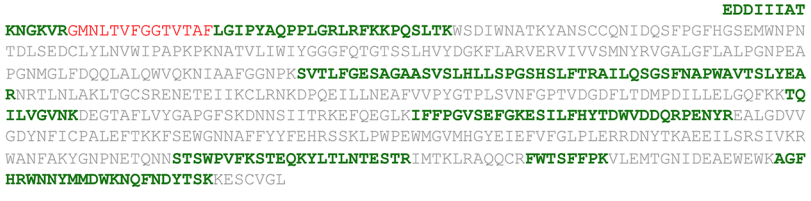

Figure 9. Mass spectrometry analysis of denatured HuBChE captured by monoclonal C191 from plasma stored at 45 °C for 3 weeks. Peptides identified with 99% confidence are colored green, with 50% confidence are colored red. The accession number for HuBChE is P06276.

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formations that have minimal activity. Monoclonal C191 recognizes a completely denatured HuBChE structure. The use of these antibodies in combination is expected to capture 95% of HuBChE from plasma stored at elevated temperatures, making it possible to determine nerve agent exposure in plasma stored under nonideal conditions.

Other monoclonal antibodies to HuBChE can be used in place of B2 18-5 to capture HuBChE from plasma. Monoclonal HAH 002-01, also called 3E8, from BioPorto Diagnostics or from Thermo Fisher has been used by the Centers for Control and Prevention (Atlanta, GA) and the Federal Medical-Biological Agency of Russia (St. Petersburg) to immunopurify HuBChE from plasma.[1,16] Monoclonal 3E8 has been used in a magnetic electrochemical sensing platform to monitor exposure to organophosphorus agents.[17]

Monoclonals mAb2 and B2 12-1 can also be used to immunopurify HuBChE from plasma,[8] although they are not commercially available. These antibodies can be made by recombinant DNA methods using the nucleotide and amino acid sequences we have deposited in the NCBI database.[8]

Monoclonals C191 and 11D8[8,18] are the only monoclonals that recognize heat-denatured HuBChE. Monoclonal C191 was made against a boiled and reduced truncated HuBChE monomer called L530stop that was missing 44 amino acids from the C-terminal tetramerization domain. The epitope for binding C191 is available not just in denatured monomeric HuBChE but also in heat-denatured tetrameric and aggregated HuBChE. Both C191 and 11D8 can be used in Western blots and ELISA. Immunohistochemical analysis of HuBChE in tissue sections fixed with paraformaldehyde has been successful with monoclonal 11D8[18] but has not been studied with C191.

The monoclonals listed above were created in mice and do not recognize mouse BChE. However, Mrvova et al.[19] created monoclonals that specifically recognize mouse BChE by immunizing BChE knockout mice with mouse BChE. Monoclonals B2 18-5 and mAb2 have broad species specificity, recognizing BChE in plasma of human, monkey, horse, cat, tiger, and rabbit.[20]

HuBChE Has Conformations Distinguishable by Monoclonal Antibodies. Native HuBChE with normal HuBChE activity is recognized by monoclonal B2 18-5. HuBChE protein with little residual activity due to heat-inactivation at 45 °C is also recognized by monoclonal B2 18-5. This monoclonal does not recognize completely misfolded HuBChE produced by heating a HuBChE solution in a boiling water bath. The selectivity of monoclonal B2 18-5 for minimally active HuBChE conformations that are different from the conformations of boiled HuBChE demonstrates the existence of several misfolded states of HuBChE.

Additional evidence for more than one misfolded HuBChE structure comes from a study that used urea-gradient polyacrylamide electrophoresis to show that tetrameric HuBChE unfolds via several partially unfolded states.[21]

Folding and Aggregation States of HuBChE. Native HuBChE in human plasma is a tetramer of four identical subunits. Each subunit contains 574 amino acids, 3 intrachain disulfide bonds, 1 interchain disulfide bond, and 9 asparagine-linked glycans. The tetramer is a dimer of dimers formed by an interchain disulfide bond at Cys 571.[22] HuBChE assembles into a tetramer via contact with polyproline-rich peptides derived from lamellipodin.[23−25] The polyproline-rich peptides interact with the tryptophan-rich C-terminus of HuBChE.[26]

Pure HuBChE heated to 100 °C releases its polyproline peptides.[23−25] We had expected the boiled HuBChE to dissociate to dimers. However, the DLS experiment and the nondenaturing gel had no hints of a HuBChE dimer. It seems the subunits reassembled into tetramers and high molecular weight aggregates by forming new disulfide bonds and by associating via hydrophobic interactions. Heat-treated pure HuBChE has been shown by others to contain high molecular weight aggregates that separate from the 340 kDa HuBChE tetramer on a size-exclusion chromatography column.[27] Heating pure HuBChE in PBS to 100 °C for 5 min or 45 °C for 9 days did not break peptide bonds.

The molten globule states of the HuBChE tetramer have been studied by subjecting HuBChE to hydrostatic pressure.[5] A pressure of 2 kbar irreversibly inactivated HuBChE without significantly changing its secondary structure. A pressure of 8 kbar completely unfolded the HuBChE tetramer but did not dissociate the tetramer. Upon release of pressure, the HuBChE protein formed aggregates with no catalytic activity. Thus, denaturation by high pressure and denaturation by high temperature yield similar misfolded HuBChE structures.

Method to Detect Inactive HuBChE in Pure HuBChE Preparations. The present study demonstrates a method for detecting inactive HuBChE in a pure human HuBChE preparation. A preparation free of inactive HuBChE has a single band on a nondenaturing polyacrylamide gel stained with Coomassie blue. However, a preparation containing a significant proportion of partly denatured HuBChE has multiple bands on a nondenaturing gel and a lower than expected activity per milligram protein. These additional bands could be interpreted as originating from contaminating proteins, when in fact they simply represent inactive HuBChE structures. Pure HuBChE intended for clinical trials in humans could be tested for the presence of aggregated HuBChE by nondenaturing gel electrophoresis.

DISCLAIMER

The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention. The use of trade names is only for identification and does not imply endorsement by the Centers for Disease Control and Prevention, the Public Health Service, or the U.S. Department of Health and Human Services.

AUTHOR INFORMATION

Corresponding Author

*E-mail: olockrid@unmc.edu (O.L.).

ORCID

Thomas A. Blake: 0000-0001-8536-9998

Oksana Lockridge: 0000-0002-8345-3640

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

DLS, dynamic light scattering; HuBChE, human butyrylcholinesterase; NCBI, National Center for Biotechnology Information; PBS, phosphate buffered saline; TBST, 50 mM Tris pH 7.4, 150 mM NaCl, 0.02% Tween-20

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